Abstract. Background: The post-translational modification of proteins, including glycosylation, is known to differ between normal and tumour cells. In this study, the expression profile of two glycosyltransferases, UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-6 (ppGalNAc-T6) and α6-sialyl-transferase-I (ST6GalNAc-I) was assessed, in a cohort of women with breast cancer. Patients and Methods: Breast cancer tissues (n=127) and normal background tissues (n=33) were collected immediately after excision during surgery. Following RNA extraction, reverse transcription was carried out and transcript levels were determined using real-time quantitative PCR and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Transcript levels within the breast cancer specimens were compared to the normal background tissues and analyzed against conventional pathological parameters and clinical outcome over a 10 year follow-up period. Results: Significantly higher levels of ppGalNAc-T6 were found in the breast cancer specimens compared to the background tissue (p=0.015). There was a non-significant trend for levels to increase with the Nottingham Prognostic Index (NPI) and TNM stage and those who died from breast cancer. ST6GalNAc-I expression was associated with better prognosis, reaching significance when comparing patients who remained disease free to those with distant recurrence (p=0.0096). The relationship approached significance when comparing NPI 2 to NPI 3 (p=0.058) and disease free patients to non-disease free patients (p=0.052) or those who died of breast cancer (p=0.060). For both enzymes a significant association with ductal type was found. Conclusion: Expression of ppGalNAc-T6 is significantly higher in breast cancer compared to 'normal'/benign breast tissue samples. ST6GalNAc-I expression in breast cancer is associated with better prognosis.

Our appreciation of the extent to which tumour cells differ from their normal counterparts has developed significantly over the last two decades, driven largely by technological improvements in analytical techniques. Genetic abnormalities have been shown to contribute to the malignant phenotype through their attendant transcriptional and translational consequences. More recently, advances in molecular biology have permitted the study of post-translational events, such as glycosylation, which have the potential to significantly modify protein structure. Such alterations can result in the expression of a variety of tumour-associated carbohydrate antigens (1). The glycoproteins of tumour cells, including secreted or cell-membrane associated mucins, show significant variation, particularly within the mucin-type O-glycans, which harbour several cancer associated epitopes including the Thomsen-Friedenreich (T) antigen, Thomsen-nouveau (Tn) antigen, sialyl-Tn (STn) antigen and certain Lewis antigens (2, 3). In addition to conferring structural changes, patterns of glycosylation can have significant and diverse implications for glycoprotein function, including: signal transduction, antigenicity and interactions with immune effector cells, cell-cell and cell-stroma adhesion, angiogenesis, invasive potential and the metastatic competence of tumour cells (4-8). Hence, the study of such post-translational modifications could provide valuable mechanistic insights, presenting opportunities for diagnostic, prognostic and potentially therapeutic applications (9-11).

The complex and subtle molecular mechanisms which influence cellular glycodynamics remain poorly understood. Glycosylation patterns are influenced by a family of glycosyltransferase enzymes, whose specificities, sequential action, relative activity levels and intracellular localization are of critical importance in determining a cell-specific O-glycosylation profile (12). Mucin type O-glycosylation begins...
with the addition of GalNAc to serine and threonine amino acids on the polypeptide by UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-T’s) which are localised throughout the golgi (13, 14). The resulting antigen, termed the Tn antigen, is then modified by the addition of sugar residues. This process is catalysed by further glycosyltransferases, through systems referred to as the core 1 or core 2 pathways, in order to generate a variety of O-glycans (15-17). Under normal conditions, the Tn moiety is then masked by subsequent sugar residues. The differential expression of ppGalNAc-T’s in cancer cells could lead to increased initiation of O-linked glycosylation with normally unoccupied potential glycosylation sites being glycosylated and cancer-associated antigens, such as Tn, emerging on the cell surface (18-21). Pathological exposure of such core region carbohydrates in cancer may reflect the deregulation of mechanisms responsible for the synthesis of the Tn antigen, or equally important, those involved with its further processing.

The ppGalNAc-T enzyme family has been implicated in the aberrant glycodynamics of several neoplasms (1). Each isoform has a different substrate specificity and expression profile. Expression and distribution studies have found the sixth member of the ppGalNAc-T family, ppGalNAc-T6, to be very restricted in normal tissue, but present in oral squamous carcinomas (22, 23). Although completely absent in healthy breast tissue, the enzyme has a high degree of homology with ppGalNAc-T3, the mRNA of which has been identified in cell lines derived from mammary gland adenocarcinomas (24). Freire et al. (25) concluded that ppGalNAc-T6 showed the greatest specificity for breast cancer, as confirmed by Brooks et al. (26).

In breast carcinomas there is a tendency for the addition of shorter O-glycans (27), which are themselves associated with increased sialylation (28), resulting in the overexpression of sialylated antigens at the surface of cancer cells. The sialyltransferases represent a group of enzymes which catalyze the biosynthesis of sialylated glycans (29). One potential substrate for this group of enzymes is the Tn antigen. Sialic acid is added in α2-6 linkage to GalNAc by α6-sialyltransferase (ST6GalNAc) (30). Of the two isoforms of this enzyme, α6-sialyltransferase I (ST6GalNAc-I) is believed to be the principal STn synthase in vivo (31). The carbohydrate antigen STn is a tumour associated disaccharide carried by mucins or within mucin like domains of several glycoproteins, including cell surface associated mucin 1 (MUC1), cluster differentiation 44 (CD44) and integrin subunits (32-35). STn expression is normally restricted to the lumen of secretory tissues and is absent from the breast (36). However, in breast cancer ST6GalNAc-I RNA is correlated with STn expression (17). STn is expressed in approximately one-quarter to one-third of breast carcinomas and is associated with metastatic competence, poor response to chemotherapy and poor prognosis (37-41). STn expression has also been reported in several other carcinomas including gastric, pancreatic, colorectal, ovarian and cervical (42-47). STn is often associated with lymph node involvement, distant metastasis, and a decreased survival of patients with gastric (48-51) or colorectal cancer (52-54). However, the molecular mechanisms through which STn enhances the malignant phenotype have yet to be elucidated.

In this study, the expression profile of ppGalNAc-T6 and ST6GalNAc-I was assessed in a cohort of women with breast cancer. Transcript levels were evaluated against established pathological parameters and clinical outcome over a 10 year follow-up period.

### Patients and Methods

**Patients and samples.** Institutional guidelines, including ethical approval and informed consent, were followed. Breast cancer tissues (n=127) and normal background tissues (n=33) were collected immediately after excision during surgery and stored at −80°C until use. A consultant pathologist examined haematoxylin and eosin stained frozen sections to verify the presence of tumour cells in the collected samples. Normal tissue was derived from the background breast parenchyma of breast cancer patients within the study group. Medical notes and histology reports were used to extract the clinicoc-
pathological data (Table I). A customized database was established to record the data.

Materials. RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, UK). The PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Sigma-Aldrich. Custom made hot-start Master mix for quantitative PCR was obtained from Abgene (Surrey, England, UK). Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5-10 mm and kept for routine histological analysis. An additional 15-20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using a reverse transcription kit with an anchored olig (dT) primer supplied by Abgene, using 1 mg of total RNA in a 96-well plate. The quality of cDNA was verified using β-actin primers (Table II).

Quantitative analysis of glycosyltransferases. The level of ppGalNAc-T6 and ST6GalNAc-I transcripts from the above prepared DNA were determined using real-time quantitative PCR based on the AmplitFluor technology, modified from a method reported previously (56). The PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACAGTACATGATGAGGGGCTTC-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added. The product expands one intron. The primers used for each glycosyltransferase enzyme are detailed in Table II. The reaction was carried out using a Hotstart Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which had the Z sequence, 10 pmol of FAM (fluorogenic reporter dye, carboxyfluorescein) tagged probe (Intergen Inc.), and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (Bio-Rad Ltd, Hemel Hempstead, England, UK), which is equipped with an optic unit that allows real-time detection of 96 reactions, under the following conditions: 94°C for 12 min and 50 cycles of 94°C for 15 sec, 55°C for 40 sec, and 72°C for 20 sec. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. The levels of ppGalNAc-T6 and ST6GalNAc-I expression were then normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was already quantified in these specimens, to correct for varying amounts of epithelial tissue between samples (57). The primers used for GAPDH are detailed in Table II. With every PCR run, a negative control without a template and a known cDNA reference sample as a positive control, were included.

Statistical analysis. The Mann-Whitney U-test and two-sample t-test were used for statistical analysis. The ppGalNAc-T6 and ST6GalNAc-I transcript levels within the breast cancer specimens were compared to normal background tissues and analyzed against conventional pathological parameters and clinical outcome over a 10 year follow-up period. In each case the true copy number was used for statistical analysis and hence the samples were not classified as positive or negative. The statistical analysis was carried out using Minitab version 14.1 (Minitab Ltd. Coventry, England, UK) using a custom written macro (Stat 2005.mtw). For purposes of the Kaplan-Meier survival analysis, the samples were divided arbitrarily into two groups, ‘high transcript level’ or ‘low transcript level’, for each glycosyltransferase enzyme. The cut-off was guided by the Nottingham Prognostic Index (NPI) value, with which the value of the moderate prognostic group was used as the dividing line at the start of the test. Survival analysis was performed using SPSS version 12.0.1 (SPSS Inc. Chicago, IL, USA).

Results

ppGalNAc-T6. The ppGalNAc-T6 expression profiles were normalised against GAPDH (Table III). ppGalNAc-T6 was found to be expressed in both normal/benign breast tissue and breast cancer specimens. Significantly higher levels were
found in the breast cancer specimens compared to the background tissue (mean copy number=10.0 vs. 0.067, p=0.015). Although there was a trend for levels to increase with NPI and TNM stage and those who died from breast cancer, this did not reach statistical significance.

In addition, a significant association with ductal type was found (mean copy number=9.8 vs. 0.050, p=0.041). However, no relationship with tumour grade or oestrogen receptor (ER) status was observed.

The overall survival curve for women with tumours which were classified as having ‘high levels’ of ppGalNAc-T6 transcript was not found to differ significantly from that of their ‘low level’ counterparts, Figure 1 (p=0.38).

**Table III. Summary of expression profiles for the overall cohort, followed by subgroup analysis for tumour specimens and benign specimens. Values represent the true copy number of mRNA transcripts (normalised against GAPDH) and are expressed as mean (range, median).**

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Tumour</th>
<th>Benign</th>
</tr>
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<tbody>
<tr>
<td>ppGalNAc-T6</td>
<td>7.68 (0-353.40, 0)</td>
<td>9.98 (0-353.40, 0)</td>
<td>0.0666 (0-1.792, 0)</td>
</tr>
<tr>
<td>ST6GalNAc-I</td>
<td>1656 (0-76648, 15)</td>
<td>1227 (0-31742, 14)</td>
<td>3257 (0-76648, 11)</td>
</tr>
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</table>

**Discussion**

In the present study, ppGalNAc-T6 expression was identified in both normal/benign breast tissue and breast cancer specimens. However, significantly higher levels were found in the breast cancer specimens compared to the background tissue. The specificity of ppGalNAc-T6 for breast cancer has been demonstrated by Freire et al. (25), who identified expression in all 3 human breast cancer cell lines evaluated and 88% of primary breast cancers. These findings have been confirmed by Brooks et al. (26) who also identified an increased range of ppGalNAc-T’s in malignant cell lines, compared to their ‘normal’/benign counterparts.

Berois et al. (12), demonstrated ppGalNAc-T6 expression in 81% of a series of breast carcinomas and 91% of ductal carcinoma in-situ (DCIS), in contrast to only 20% of benign lesions and none of the five normal breast tissue samples. Furthermore, a significant association with T1 stage was found, suggesting that up-regulation may represent an early event. Expression was shown to continue in aggressive and metastatic lesions, however, no correlation with nodal status or histological grade was identified (12). ppGalNAc-T6 has also been demonstrated in bone marrow aspirates from breast cancer patients, where expression was significantly associated with early recurrence (25). Hence, ppGalNAc-T6 could offer prognostic utility in addition to conventional markers. In this study, although the ppGalNAc-T6 levels tended to increase with NPI and TNM stage and in those who died from breast cancer, no significant differences were identified in the overall survival curve for women with tumours which were classified as having ‘high levels’ of ppGalNAc-T6 transcript compared to their ‘low level’ counterparts, Figure 1.

In the present study, ST6GalNAc-I expression was found to be associated with better prognosis, this reached statistical significance when comparing patients who remained disease free to those with distant recurrence and approached significance when comparing NPI 2 to NPI 3 and disease free patients to non-disease free patients (mean copy number=1640 vs. 381, p=0.052) or those who died of breast cancer (mean copy number=1640 vs. 398, p=0.060).

In addition, a significant association with ductal type was found (mean copy number=1397 vs. 51, p=0.014). However, no relationship with tumour grade or ER status was observed. Within the ductal carcinoma subgroup, high expression remained associated with favourable prognosis and reached statistical significance when comparing patients who remained disease free to those with distant recurrence (mean copy number=1855 vs. 0.138, p=0.018).

The overall survival curve for women with tumours which were classified as having ‘high levels’ of ST6GalNAc-I transcript was not found to differ significantly from that of their ‘low level’ counterparts, Figure 2 (p=0.8).
other mechanisms. In keeping with this, Tn and STn expression in cervical cancer specimens, colon cancer and melanoma derived cell lines have recently been found to be associated with loss-of-function somatic mutations in the Cosmc gene. This single exon gene encodes a molecular chaperone required for formation of the active T-synthase (42). Hence, specific genetic mutations within glycosylation pathways can directly influence expression of these antigens across a range of different neoplasms.

Breast cancer cell lines transfected with ST6GalNAc-I show significantly altered patterns of O-glycosylation and exhibit enhanced tumourigenicity, in terms of decreased adhesion, increased migration and increased growth (33, 58, 61-63). Similarly, STn expression is associated with decreased cell adhesion on extracellular matrix components and increased cell migration (33). STn bearing mucins may also interfere with cancer cell recognition by the immune system and natural killer cell function, perhaps protecting blood borne metastatic cells from degradation (64). Furthermore, anti-STn-mAb/STn-bearing protein immunocomplexes enhance vascular endothelial growth factor secretion by tumour-infiltrating macrophages, improving angiogenesis (65). STn expression has also been associated with the invasiveness of ovarian carcinomas, with enhanced expression in the invasive front (66-68). Davidson et al. also found that the expression of STn of distant metastases appeared to be significantly lower than that of the primary ovarian tumour, suggesting that such changes may be transient and play a greater role at particular stages of the metastatic pathway, for example, facilitating the dissociation of cancer cells from the primary tumours.

STn expression has been associated with poor prognosis in a range of solid human carcinomas and appears to identify patients that may be relatively refractory to conventional treatment (52, 53, 69). The selective expression of STn has been considered for its therapeutic utility (70-74). Cancer vaccine studies have also identified STn as a potential target in breast and ovarian carcinomas (75, 76). In addition to the differential expression of STn between normal tissue and carcinomas, altered expression has also been observed in pre-malignant lesions of the gastrointestinal tract, such as intestinal metaplasia (50), adenomatous polyps (77) and chronic ulcerative colitis (78). Hence, STn is likely to have an important role to play in the development and progression of the malignant phenotype.
Limitations of the present study included the use of background parenchyma from breast cancer patients to provide ‘normal tissue’ for comparison. Ideally, such material should be derived from patients without breast cancer in order to avoid any ‘field change’ which may exist within cancer bearing tissues. Although the sample size and follow-up period were substantial, it is possible that a larger cohort may have influenced several results which approached, but failed to reach, statistical significance. Finally, in addition to the measurement of mRNA transcript levels, quantitative analysis of enzyme, Tn and STn expression should be undertaken to ensure concordance.

Conclusion

The expression of ppGalNAc-T6 is significantly higher in breast carcinomas compared to ‘normal/benign breast tissue samples and ST6GalNAc-I expression is significantly associated with better prognosis. Further studies are required to elucidate their contribution the development and progression of the malignant phenotype.

References

74 Holmberger LA, Opalin DV, Gooley T and Sandmaier BM: The role of cancer vaccines following autologous stem cell rescue in breast and ovarian cancer patients: experience with the STn-KLH vaccine (Theratope(R)). Clin Breast Cancer 3: S144-151, 2003.

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